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Cryohemolysis for the Detection of Hereditary Spherocytosis: Correlation Studies With Osmotic Fragility and Autohemolysis

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Laboratory methods aimed to assess the presence of spheroidal cells such as osmotic fragility, autohemolysis, and glycerol lysis time are very elaborate, time consuming, and often give inconclusive results. We have developed a diagnostic test based on a unique sensitivity of HS cells to hypertonic cryohemolysis and analyzed blood samples of 55 HS patients. The patients were divided into two subgroups, clinically affected probands and their relatives. To get quantitative comparisons with the classic methods, the cryohemolysis results were compared to two parameters of the osmotic fragility test: the salt concentration that causes 50% hemolysis, and the percent lysis at a constant salt concentration. Autohemolysis results were also compared. To evaluate which of these tests has the best analytical power, we calculated the mean results and 2 SDs of each parameter in a control group, and then looked to see which of them was best in identifying the patients. The cryohemolysis test was the single parameter that identified all cases including asymptomatic carriers of the disease. The ability of this test to identify the less severe cases probably reflects the dependency of the cryohemolysis on factors that are more related to the primary membrane molecular defects and less by the surface area to volume ratio. Am. J. Hematol. 58:206–212, 1998.

Key words: autohemolysis; cryohemolysis; diagnosis; hereditary spherocytosis; osmotic fragility; skeletal interactions

INTRODUCTION

The marrow of Hereditary Spherocytosis (HS) patients produces cells with normal biconcave shape but because of a defect in their skeleton they lose membrane as they circulate through the spleen [1]. The consequence of this membrane loss is the appearance of spheroidal cells with reduced surface area to volume ratio. The assessment of this ratio is the basis for the osmotic fragility (OF) measurements, the classic way to detect HS [2]. Along with the osmotic fragility test, many laboratories perform autohemolysis measurements, in which the need for higher amounts of glucose to maintain the HS cells' integrity is expressed [2]. Both tests are cumbersome and time consuming and too often do not give conclusive results. An alternative method, the glycerol lysis time [3], which also depends on the cells' surface area to volume ratio, has not been widely accepted.

In 1990 we suggested [5] a new method for the diagnosis of HS patients that does not necessarily depend on erythrocyte surface area to volume ratio. This test is based on our previous observation that while suspended in hypertonic solutions, HS red cells are much more susceptible to temperature changes than normal cells [6].

Since the introduction of this procedure in the laboratory, we have analyzed blood samples of 55 patients. Comparison of OF and autohemolysis tests to the cryohemolysis on a quantitative basis further shows the analytical power of this procedure.

MATERIALS AND METHODS

Patients

All patients were referred to us because of a Coombs' negative hemolytic anemia. Laboratory findings were high reticulocyte counts, increased indirect bilirubin, and

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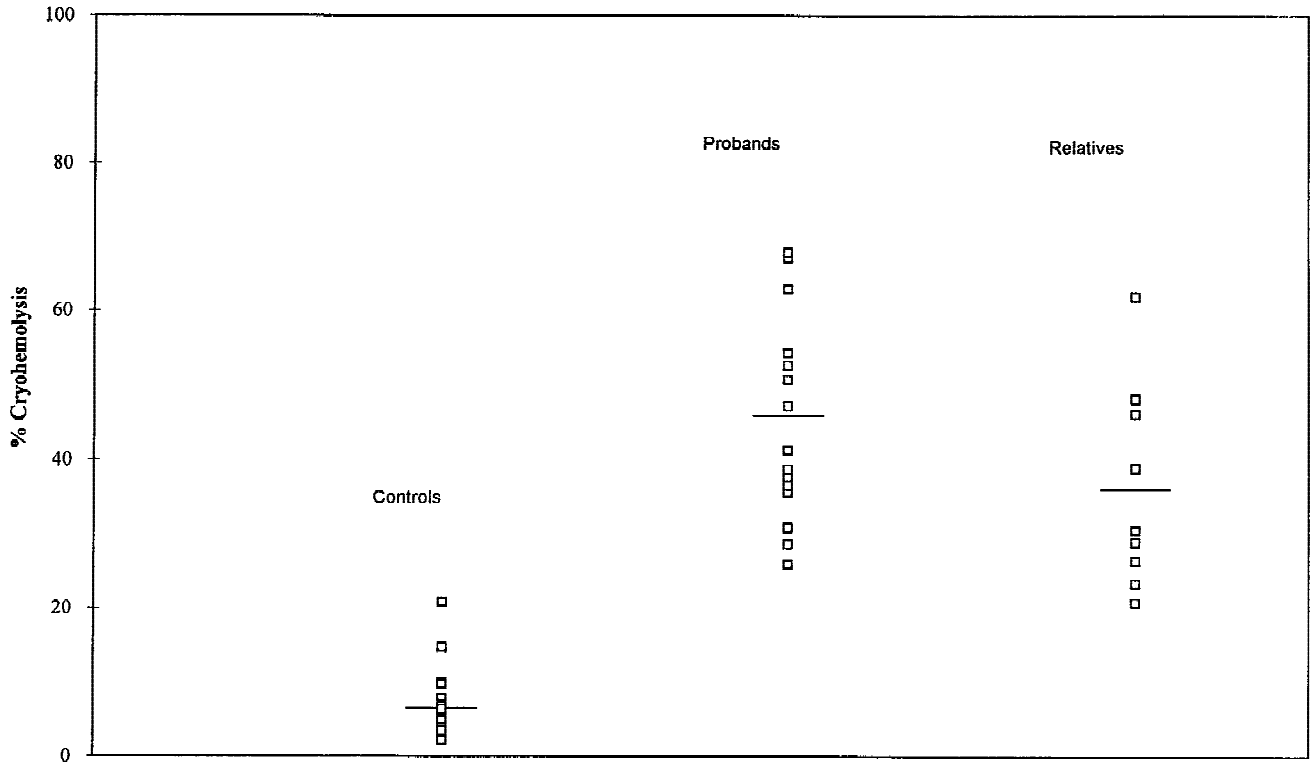

Fig. 1. Cryohemolysis levels subdivided into HS probands, relatives, and healthy controls.

absence of free haptoglobin. Most cases had enlarged spleens of varying degrees. The diagnosis of HS was based on osmotic fragility measurements of fresh and incubated blood, autohemolysis tests, and spherocytes in peripheral blood smears. Family members of the probands were examined via the same assays and those who presented at least signs of hemolysis were considered carriers of the disease. Some had a history of fluctuating jaundice, gallstones, or occasional anemia following infections. Two subjects were considered carriers although they were completely asymptomatic (see Table I and Discussion), apart from reduced haptoglobin in one of them.

Osmotic Fragility and Autohemolysis

Both fresh and sterilely incubated blood at 37°C for 24 h were performed according to Dacie and Lewis [2].

Cryohemolysis (Based on Our Previous Report [5])

Following are brief instructions.

Blood. Three milliliters in standard hematology vacutaines (K_3EDTA) freshly drawn or 1 day old.

Reagents.

1. Cold saline
2. Deionized water
3. Buffered 0.7M sucrose (21.47 g sucrose in 50 mM phosphate buffer pH7.4)[1]

Equipment.

1. Water bath at 37°C
2. Ice-cold water controlled at 0°C
3. Clinical centrifuge
4. Spectrophotometer (540 nm)
5. Vortex, Stop watch, Pipettors

Method.

1. Wash cells three times with cold saline. Keep final suspension (50–70% packed cells) on ice until tested.
2. For each patient and for control cells, prepare 2-ml duplicates of buffered sucrose. Keep 10 min at 37°C for temperature equilibration.
3. Pipette 50 μl of the washed packed cells to each of the duplicate tubes containing the warmed buffered sucrose, vortex immediately for few seconds, and incubate 10 min at 37°C.[2]

[1]It is recommended to freeze 2-ml aliquots in tubes ready to be used.
[2]It is important to keep measured time intervals between pipetting the blood samples of each patient. The same time intervals should be kept in the subsequent steps.

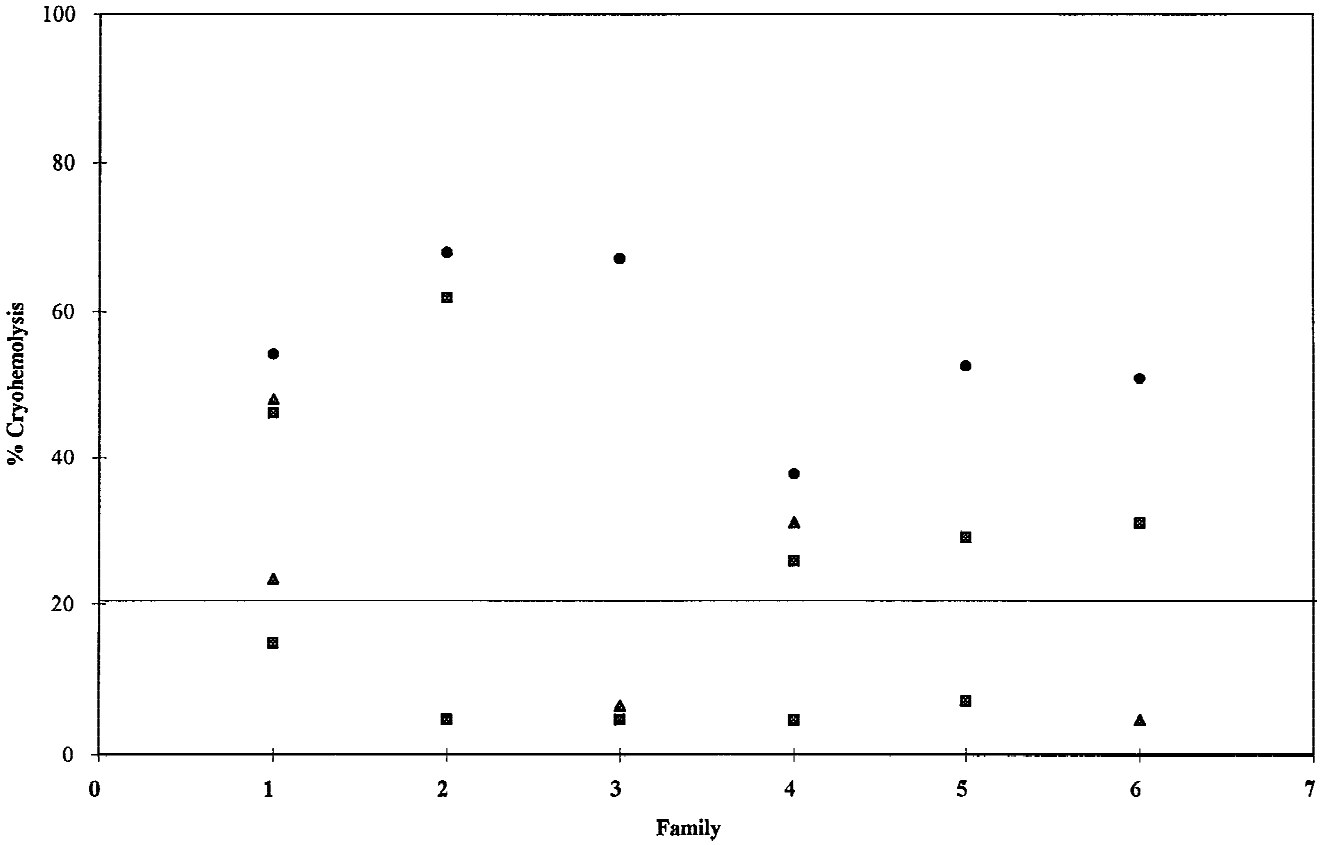

Fig. 2. Cryohemolysis levels presented for members of 6 families. ● probands; ▨, parents; ◬, siblings.

4. After exactly 10 min at 37°C, transfer the tubes to an ice-cold bath for another 10 min.
5. After exactly 10 minutes at 0°C, vortex for few seconds and centrifuge to sediment the remaining cells.
6. Read the absorbance of supernatant at 540 nm (if needed, dilute 200 μl in 4 ml water).
7. To get a 100% hemolysis, pipette 50 μl of each sample into 2 ml deionized water (make duplicates). Centrifuge and dilute 200 μl of supernatant in 4 ml water. Read absorbance at 540 nm.

Calculation. % cryohemolysis = OD540 test/OD hemolysate × 5.

Normal range. 3%–15%.

Hereditary spherocytosis. >20%.

RESULTS

The mean value of cryohemolysis detected in 55 patients is 42 ± 13% compared to 7.0 ± 4.4% in 42 healthy controls ($P < 0.001$). Out of the 55 cases, we retrieved the data of the recently analyzed 24 patients. Clinically they were divided into two subgroups: clinically affected probands and their family relatives. Figure 1 shows the distribution of the levels of cryohemolysis measured for 15 probands, 9 family relatives, and 12 healthy individuals analyzed in parallel. As seen, both the more severely affected and their relatives have results distinctly apart from the controls. The more affected had a mean of 45 ± 13% ($P < 0.001$), and their family relatives had a mean of 36 ± 13% ($P < 0.001$), compared to the healthy controls who had a mean of 7.0 ± 5.1%. The slight difference between the two patient subgroups is statistically insignificant. Figure 2 summarizes the cryohemolysis data of 6 families, demonstrating the power of the cryohemolysis test to detect carriers of the disease. As seen in Figure 2, the probands had always high values of cryohemolysis and in 5 out of the 6 families we detected either a parent and/or sibling with similar elevated results. The fact that levels of cryohemolysis are not evenly distributed among HS patients reflects the heterogeneity of this disease. Differences within some of the families are probably due to unidentified factors that modulate the expression of the underlying molecular defects. The patient of the sixth family (family no. 3 in Fig. 2) has a severe chronic hemolysis (two postnatal exchange transfusions were given) and he is also mentally retarded. His father is not available for cryohemolysis studies but all other hematological tests were normal. At this point we cannot know whether the patient has an autosomal dominant, recessive form, or a new mutation of the disease. Although the OF

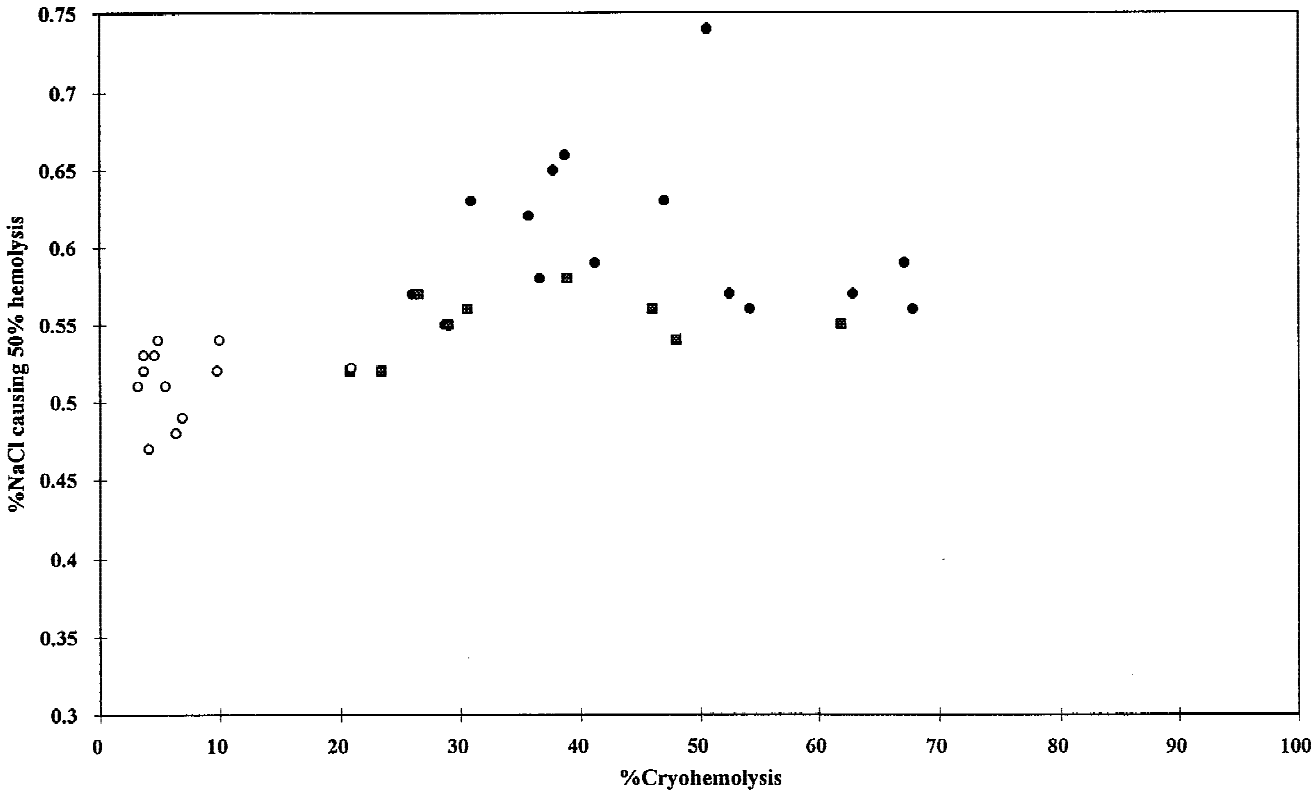

Fig. 3. NaCl concentrations that cause 50% osmotic lysis vs. the extent of cryohemolysis: ●, 15 probands (mean: 0.60 ± 0.05); ▨, 9 relatives (mean: 0.55 ± 0.02); ○, 12 controls (mean 0.51 ± 0.02). Coefficient of correlation $R^2 = 0.3298$.

test gives results that are interpreted as either normal or abnormal, attempts were made by Eber et al. [7] to correlate the results with the extent of spectrin deficiency and the severity of the disease. In their study, the authors could show that patients with moderate or severe disease could be distinguished from trait or mildly affected patients by measuring differences in spectrin content and to some extent they could correlate them with OF and autohemolysis.

In the present study we tried to correlate the extent of cryohemolysis with the OF results on a quantitative basis. For this purpose we selected two parameters from the OF curve of each patient: (1) the salt concentration that causes 50% hemolysis (some laboratories use it as a screening tool); (2) the percentage of osmotic lysis that occurs at a certain salt concentration. Figures 3 and 4 summarize these two parameters as detected in blood after incubation in correlation with the cryohemolysis results of the same patients. As seen in Figure 3, a very poor coefficient of correlation ($r^2 = 0.3298$) is found when the salt concentration, which causes 50% osmotic lysis, is compared to the extent of cryohemolysis. A better correlation ($r^2 = 0.6114$) is obtained when the extent of osmotic lysis observed at 0.55% NaCl is compared to cryohemolysis (Fig. 4). This particular salt concentration was chosen because it gave the best discriminating results between normal and HS samples (see Table I). Similar studies with fresh specimens gave much poorer correlation between OF and cryohemolysis results (not shown). Autohemolysis, which is still being used in some laboratories, was also compared to the cryohemolysis (Fig. 5) and as well gave poor correlation ($r^2 = 0.3929$). The diagnostic power of the cryohemolysis is clearly demonstrated in Table I. Here we first calculated the mean results and 2 SD above parameters of the 12 control specimens (see legends to Figs. 3–5); any result that exceeds this value is considered as a diseased state. Table I includes data of fresh and incubated cells and, as expected, the OF results of the incubated blood are much more indicative for the presence of the disease than those of the fresh blood. But even with the incubated blood, the screening for salt concentration, which causes 50% lysis, identified 14 out of the 15 probands, and only 4 out of their 9 family relatives. The OF at 0.55% NaCl seems to be a much better parameter; it identified all probands, but failed to identify 2 obligatory carriers of the disease. The only test that detected all the HS cases is the cryohemolysis test. It is important to note here that the diagnosis of the two carriers that were identified only by the cryohemolysis (one had reduced haptoglobin, otherwise both had no clinical or laboratory signs of hemolysis), was established through their family relations: One is a

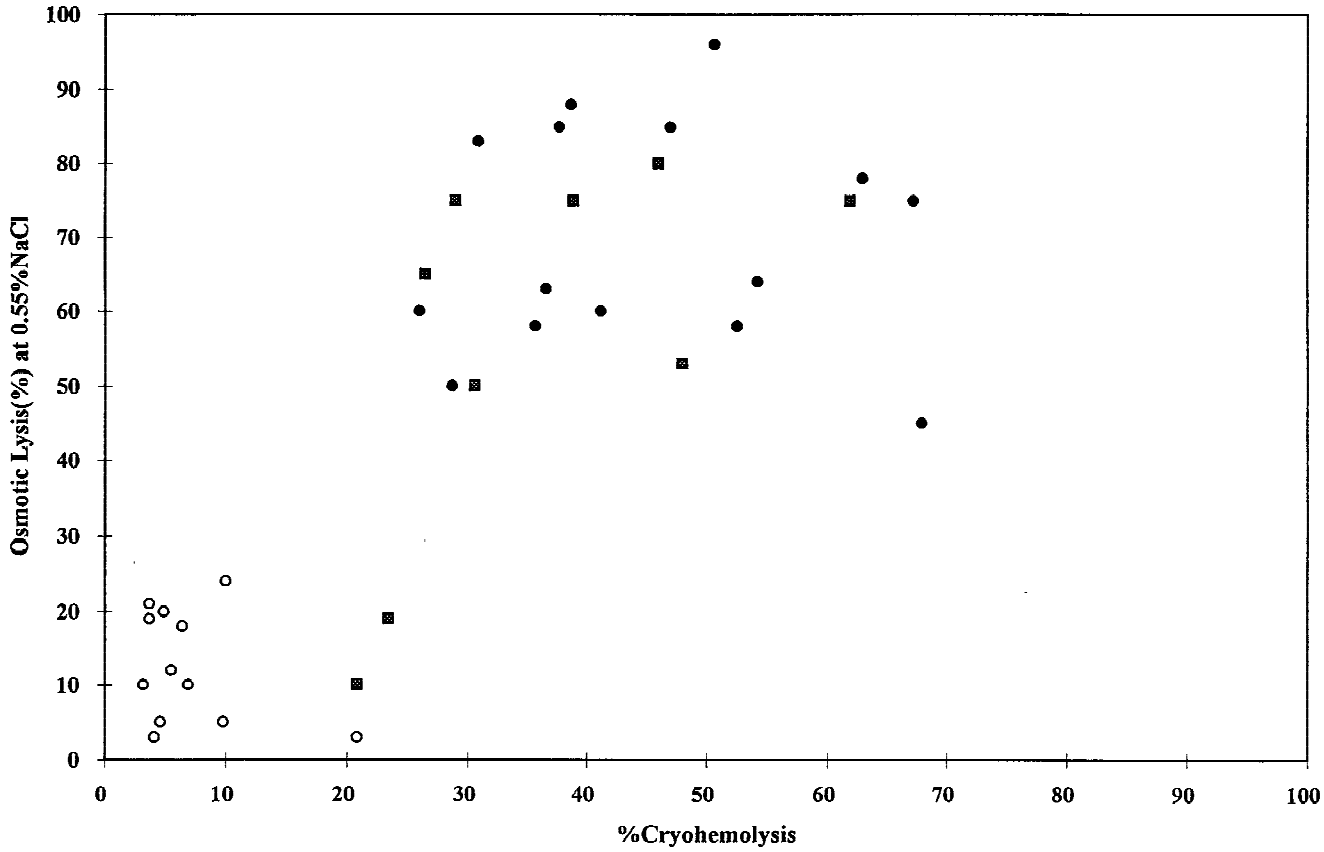

Fig. 4. Osmotic lysis at 0.55% NaCl vs. cryohemolysis. ●, 15 probands (mean: 70 ± 15); ▨, 9 relatives (mean: 56 ± 26); ○, 12 controls (mean: 13 ± 8). Coefficient of correlation $R^2 = 0.6114$.

mother of two affected children (not presented in Fig. 2) and the second is a brother of three affected siblings (family 1 in Fig. 2).

DISCUSSION

When red cells are cooled from 37° to 0°C, while suspended in hypertonic medium, they undergo massive hemolysis. The extent of hemolysis depends on the tonicity and the temperature of the medium. This phenomena was first described by Green and Jung [8] who suggested that under these circumstances the cells' lipid bilayer undergoes phase transition from fluid to gel state. Dubbelman et al. [9] further studied this phenomenon and suggested that under hypertonic conditions, because of the cells' shrinkage, the membrane cannot deal with the required mechanical accommodation and as a consequence rupture of the membranes occurs. We studied the hypertonic cryohemolysis of pathological cells, and found that HS erythrocytes are uniquely sensitive under these conditions [6], proposing to use this unique sensitivity as a tool for the detection of this disease [5]. Our observations were confirmed by Melrose [10] who studied several hemolytic states and found that apart from the HS patients, Melanesian type of elliptocytosis have also increased cryohemolysis. Lately, the usefulness of this method to detect HS was also confirmed by Remero et al. [11] who compared a group of patients with clinical HS to patients with mechanical heart valve prothesis and to malignant hematological diseases and healthy blood donors. Genetically, HS is a heterogeneous disease [1]. The fact that all our cases were susceptible to hypertonic cryohemolysis probably means that it depends on a common skeleton distortion secondary to the primary underlying different defects. It seems that under hypertonic conditions, the shrunken HS cells are less capable of coping with the necessary changes associated with lipid phase transitions that occur upon cooling. Up till now the molecular defects recognized in HS are associated with proteins anchoring the skeleton framework to membrane. Recently, Hassoun et al. [12] described six frameshift and nonsense mutations and four missense mutations of the β spectrin gene in 11 unrelated families out of 40 with spectrin or spectrin and ankyrin deficiencies. Mutations of band 3, ankyrin, and protein 4,2 have also been reported [1]. We have postulated that the strains imposed on the skeleton during the temperature changes expose defects in those proteins that are involved in the vertical

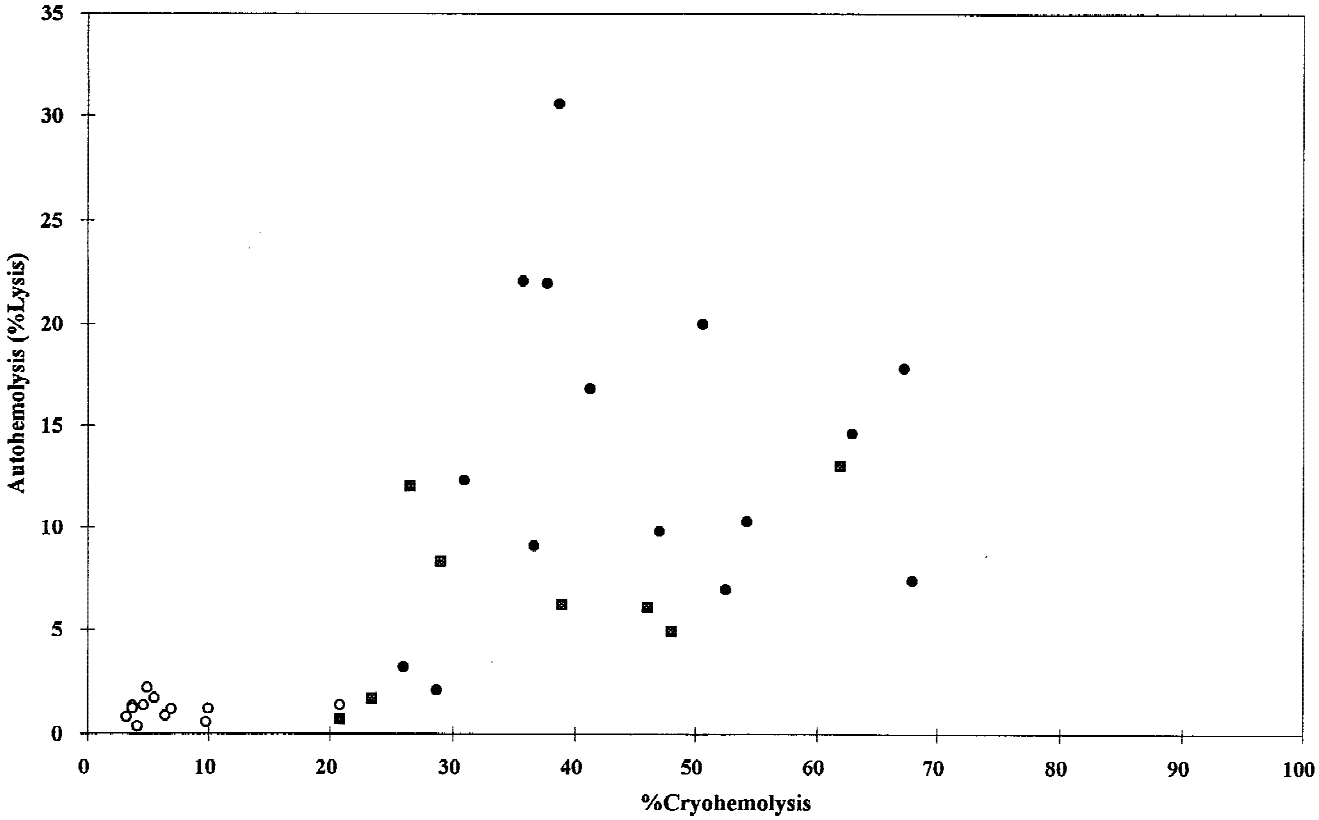

Fig. 5. Autohemolysis vs. cryohemolysis. ●, 15 probands (mean: 13.7 ± 7.9); ▨, 8 relatives (mean: 6.6 ± 4.4); ○, 12 controls (mean: 1.2 ± 0.5). Coefficient of correlation R^2 = 0.3929.

TABLE I. Ability of OF Parameters and Cryohemolysis Test to Identify the Disease in 24 HS Cases (15 Probands + 9 Relatives)

	Positive results		
Method	Relatives (9)	Probands (15)	False negative
Fresh blood			
% NaCl causing 50% lysis	2	7	15
Osmotic lysis at 0.55% NaCl	4	12	8
Incubated blood			
% NaCl causing 50% lysis	4	14	6
Osmotic lysis at 0.55% NaCl	7	15	2
Cryohemolysis	9	15	0

interskeletal interactions [5]. The finding that Melanesian type of HE are also sensitive to this test [10] may indicate a possible role of band 3 fragment, missing in these types of cells [13], in the above-mentioned vertical interactions. One family with spherocytic HE and few HEMPAS patients also had increased levels of cryohemolysis [5].

Close association between the primary membrane defects and the susceptibility to hypertonic cryohemolysis can explain the superiority of this test in identifying carriers in whom the surface area to volume ratio is not reduced enough to be detected by the OF tests (Table I). It is still possible that the reduced surface area to volume ratio present in HS cells has some contribution to their increased susceptibility. Such an assumption can explain the correlation, although quite poor, with the OF measurements and the severity of the disease (Figs. 1–5). But, as was shown in our previous report [5], artificially induced (by chemicals or by heat) spherocytes, which are osmotically very fragile, have normal levels of cryohemolysis. This indicates that reduction in the ratio between surface area and volume alone is not enough to affect the extent of cryohemolysis. As was also indicated in that report, this test has a 100% sensitivity and a quite high specificity (90% in relation to the general population of hospitalized patients and 87% in relation to autoimmune hemolytic anemia).

The data in the present report show the superiority of the cryohemolysis over other existing procedures in identifying HS cases. Its high diagnostic power together with very low cost, simple procedure, and quick results make this test the right choice for the purpose of diagnosing HS patients.

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